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Molecular Phylogeny of *Trachelospermum* (Apocynaceae) in Japan Based on cpDNA and nrDNA Nucleotide Sequences

AZUSA UEMACHI* AND TAKASHI SHIMOMURA

*Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Simogamo-hangicho 1-5, Sakyo, Kyoto 606-8522, Japan. *qm4t-uemc@asahi-net.or.jp (author for correspondence)*

A molecular phylogenetic analysis of *Trachelospermum* (Apocynaceae; *T. asiaticum*, *T. jasminoides* var. *pubescens* and *T. gracilipes* var. *liukiuense*) in Japan was conducted using cpDNA and nrDNA nucleotide sequences. The cpDNA phylogeny did not agree with morphology-based taxonomy, whereas the ITS phylogenies agree with morphology. These results can be explained by chloroplast capture or incomplete lineage sorting. The molecular data also indicate sequence differentiation in *T. gracilipes* var. *liukiuense* between the Bonin and Ryukyu islands. Furthermore, in the ITS sequences of *T. jasminoides* var. *pubescens*, additive nucleotide sites were present in the substitution sites between *T. asiaticum* and *T. jasminoides* var. *pubescens*. The results support the possibility of natural hybridization between *T. asiaticum* and *T. jasminoides* var. *pubescens*.

Key words: chloroplast DNA, hybridization, ITS, molecular phylogeny, *Trachelospermum*

Species of *Trachelospermum* Lem. (Apocynaceae) are evergreen, woody lianas native to southern and eastern Asia with one species in southeastern North America. Three species occur in Japan (Yamazaki 1993). *Trachelospermum asiaticum* (Siebold & Zucc.) Nakai occurs westward from Iwate prefecture. *Trachelospermum asiaticum* has glabrous leaves and the apex of the anther is exserted from the corolla throat. Plants with larger leaves and growing in coastal areas have been treated as var. *majus* (Nakai) Ohwi (Ohwi 1965, Kitamura & Murata 1971, Satake *et al.* 1989), but varietal distinction was not recognized by Yamazaki (1993) in his treatment for the Flora of Japan. In this study, we recognize var. *majus* and treat samples of *T. asiaticum*, except for var. *majus*, as var. *asiaticum*.

Trachelospermum jasminoides (Lindl.) Lem. occurs from Kinki district westward in Japan. Its anthers are included within the corolla tube. *Trachelospermum jasminoides* with hirsute leaves has been separated as var. *pubescens* Makino from var. *jasminoides*, which occurs in China and Taiwan and has glabrous leaves (Ohwi 1965, Kitamura & Murata 1971, Satake *et al.*, 1989). Yamazaki (1993), however, suggested that var. *pubescens* is not distinct from var. *jasminoides*. In this study, we recognize the plants in Japan with hirsute leaves as var. *pubescens* and those from China with glabrous leaves as var. *jasminoides*, according to Ohwi (1965) and Satake *et al.* (1989).

Trachelospermum gracilipes Hook. f. var. *liukiuense* (Hatus.) Kitam. is native to the Bonin (Ogasawara) Islands and Nansei Islands southward from Yakushima (Yamazaki 1993). As with *T. asiaticum*, *T. gracilipes* var. *liukiuense* has glabrous leaves and the apex of anthers is exserted from the corolla throat. The corolla of *T. gracilipes* var. *liukiuense* is smaller and the pedicels are shorter than in *T. asiaticum*. Nakai (1922) recognized *T. foetidum* (Matsum. & Nakai) Nakai as a species endemic to the Bonin Islands. Hatsusima (1940), in reviewing Japanese *Trachelospermum*, proposed a new species, *T. liukiuense* Hatusima, from Yakushima, the Amami Islands, and

Ryukyu Islands. Kitamura (1962) subsequently reduced *T. liukiuense* to a variety of *T. gracilipes* based on its morphological resemblance to *T. gracilipes* from Taiwan. *Trachelospermum gracilipes* is distributed from northeastern India through China to Korea and Taiwan (Li & Huang 1998). *Trachelospermum gracilipes* and *T. asiaticum* are not distinguished from each other in China (Jussieu 1995) and Taiwan (Li & Huang 1998). Recently, *T. foetidum* has been treated as a synonym of *T. gracilipes* var. *liukiuense* (Yamazaki 1993).

Because of the different opinions regarding the taxonomic treatment of Japanese *Trachelospermum* and the lack of a phylogenetic assessment of their relationships, we conducted molecular analyses of the nucleotide sequences of the cpDNA (*trnL* intron and *trnL–F* spacer, *rpl16* intron, *rps16* intron) and nrDNA (ITS regions) to determine the taxonomy of the genus and the relationships of the taxa.

Materials and Methods

Plant materials for molecular studies

Twenty-two accessions of five taxa were collected for this study (Table 1). All specimens except for *Trachelospermum jasminoides* var. *jasminoides* were from wild individuals growing in Japan. Geographic information for the collections is shown in Fig. 1. The plants of *T. jasminoides* var. *jasminoides* were collected from the Ghent University Botanical Garden, Belgium (originally from the Hangzhou Botanical Garden, China), and from two nurseries in Japan. *Chonemorpha fragrans* (Moon) Alston was selected as an outgroup. *Chonemorpha* and *Trachelospermum* are classified in the same tribe, Apocyneae and are considered to be close relatives (Simoes *et al.* 2006, Livshults *et al.* 2007). Voucher specimens were deposited in the herbarium of Kyoto University Museum (KYO).

DNA extraction

Leaf samples were collected in the field or from cultivated plants for DNA extraction. Total genomic DNA was isolated from approximately 200 mg of leaf tissue. The protocol used was based on the cetyltrimethylammonium bromide (CTAB) method (Doyle & Doyle 1987) with slight modifications. DNA samples were purified using Wizard Minipreps DNA Purification Resin (Promega, Wisconsin, USA) and dissolved in TE buffer (10 mM Tris-HCl, 1mM EDTA, pH8.0) to a final concentration of 5 ng/μL.

Sequencing of chloroplast DNA and nuclear DNA

The non-coding regions of chloroplast DNA (*trnL* intron and *trnL–F* spacer, *rpl16* intron, *rps16* intron) and nuclear ITS regions (ITS1 and ITS2) were amplified by PCR and their nucleotide sequences were determined. PCR was performed in a total volume of 50 μL that contained: 1 unit of HotStar Taq DNA polymerase (Qiagen, Hilden, Germany), 10 × HotStar taq PCR buffer (Qiagen), 1.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 12.5 ng of genomic DNA, 0.5 μM of each primer and nuclease-free water. Primer information is presented in Table 2. Primers for the *trnL* intron and *trnL–F* spacer were newly designed in this study using published sequences of *Trachelospermum* (accession number AF214439, Potgieter & Albert 2001; accession number AB178632, Aoki *et al.*, 2004). The cycling program for three regions of cpDNA consisted of initial denaturation at 95°C for 15min, followed by 34 cycles of denaturation at 94°C for 20s, annealing at 60°C for 20s and extension at 72°C for 1.5 min, and ended with 2 min at 72°C. For nuclear ITS regions, cycling conditions were 15 min at 95°C, then 34 cycles of 20s at 94°C, 20s at 58°C and 1 min at 72°C, and finally 2 min at 72°C. PCR products were purified using Wizard Minipreps DNA Purification Resin (Promega, Wisconsin, USA) and electrophoresed in 1.0% agarose gels to confirm a single product.

For nucleotide sequencing, the BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, USA) was used on a GeneAmp9700 thermal cycler (Applied Biosystems) following the manufacturer's protocol. We used the same primers as those used for amplification. Reaction mixtures were analyzed by a 3130XL Genetic Analyzer or a 3730XL DNA Analyzer (Applied

TABLE 1. Collection details of samples used in molecular analyses.

Sample name	Wild / cultivated	Source	Voucher	Accession No.			
				ITS	*trnL* intron/ *trnL–F* spacer	*rpl16* intron	*rps16* intron
Tracheloseprmum asiaticum var. *asiaticum*-1	wild	Yuzamachi, Yamagata, Japan	AU-W34-1	AB710122	AB710074	AB710090	AB610200
T. asiaticum var. *asiaticum*-2	wild	Tateyama, Chiba, Japan	AU-W27-1	AB710123	AB710075	AB710091	AB710107
T. asiaticum var. *asiaticum*-3	wild	Kamakura, Kanagawa, Japan	AU-W29-1	AB610207	AB610186	AB610193	AB610199
T. asiaticum var. *asiaticum*-4	wild	Atami, Shizuoka, Japan	AU-W31-1	AB710124	AB710076	AB710092	AB710108
T. asiaticum var. *asiaticum*-5	wild	Hikone, Shiga, Japan	AU-W14-1	AB710125	AB710077	AB710093	AB710109
T. asiaticum var. *asiaticum*-6	wild	Nanbucho, Tottori, Japan	AU-W11-1	AB710126	AB710078	AB710094	AB710110
T. asiaticum var. *asiaticum*-7	wild	Bizan, Tokusihma, Japan	AU-W09-1	AB710127	AB710079	AB710095	AB710111
T. asiaticum var. *asiaticum*-8	wild	Chikushino, Fukuoka, Japan	AU-W44-1	AB710128	AB710080	AB710096	AB710112
T. asiaticum var. *majus*-1	wild	Kushimoto, Wakayama, Japan	AU-W02-1	AB710129	AB710081	AB710097	AB710113
T. asiaticum var. *majus*-2	wild	Nokonoshima, Fukuoka, Japan	AU-W45-1	AB710130	AB710082	AB710098	AB710114
T. jasminoides var. *pubescens*-1	wild	Kyoto Kita-ku, Kyoto, Japan	AU-W07-1	AB710131	AB710083	AB710099	AB710115
T. jasminoides var. *pubescens*-2	wild	Takatsuki, Osaka, Japan	AU-W33-1	AB710132	AB710084	AB710100	AB710116
T. jasminoides var. *pubescens*-3	wild	Kinokawa, Wakayama, Japan	AU-W15-1	AB710133	AB710085	AB710101	AB610202
T. jasminoides var. *pubescens*-4	wild	Soja, Okayama, Japan	AU-W23-1	AB610208	AB610187	AB610194	AB610201
T. jasminoides var. *pubescens*-5	wild	Chikushino, Fukuoka, Japan	AU-W35-1	AB710134	AB710086	AB710102	AB710117
T. jasminoides var. *jasminoides*-1	cultivated	uncertain, collected from a nursery	AU-R07-1	AB610209	AB610189	AB610195	AB610203
T. jasminoides var. *jasminoides*-2	cultivated	uncertain, collected from a nursery	AU-R08-1	AB710135	AB710087	AB710103	AB710118
T. jasminoides var. *jasminoides*-3	cultivated	Hanzhou Botanical Garden, China	AU-S03-1	AB710136	AB610188	AB710104	AB710119
T. gracilipes var. *liukiuense*-1	wild	Hahajima, Ogasawara, Japan	AU-W43-1	AB610211	AB610191	AB610197	AB610205
T. gracilipes var. *liukiuense*-2	wild	Hahajima, Ogasawara, Japan	AU-W43-3	AB710137	AB710088	AB710105	AB710120
T. gracilipes var. *liukiuense*-3	wild	Nago, Okinawa, Japan	AU-W42-1	AB710138	AB710089	AB710106	AB710121
T. gracilipes var. *liukiuense*-4	wild	Onnason, Okinawa, Japan	AU-W01-1	AB610210	AB610190	AB610196	AB610204
Chonemorpha fragrans (outgroup)	cultivated	Higashiyama Botanical Garden, Japan	AU-C01-1	AB710139	AB610192	AB610198	AB610206

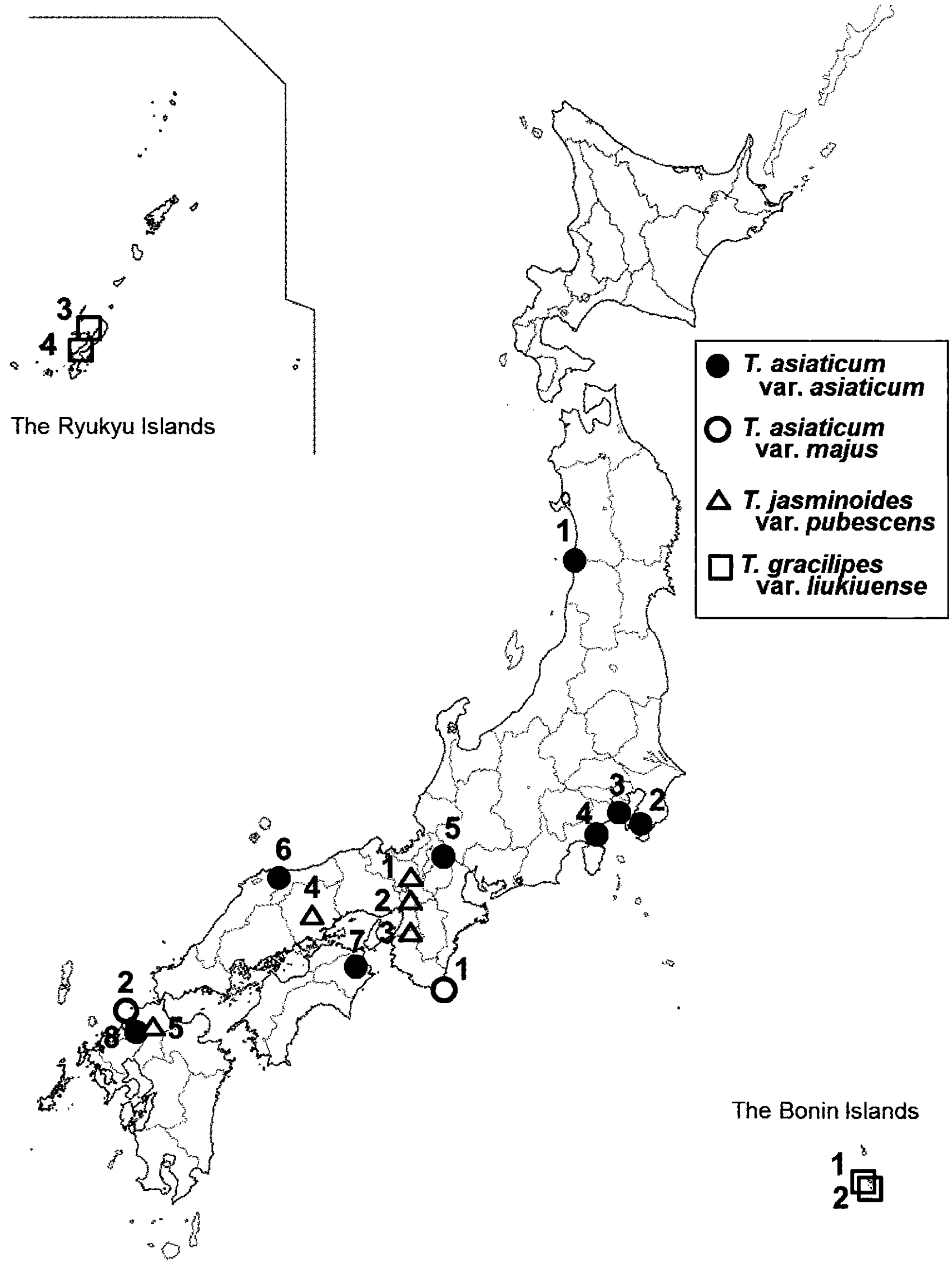
The Ryukyu Islands
3
4
● T. asiaticum var. asiaticum
○ T. asiaticum var. majus
△ T. jasminoides var. pubescens
□ T. gracilipes var. liukiuense
1
2
3
4
5
6
7
8
1
2
3
4
5
1
2
The Bonin Islands
1
2

FIG. 1. Locations from which samples of *Trachelospermum* were collected in Japan. Sample names correspond to those in Table 1.

TABLE 2. Primers used in PCR and sequencing. All primers are given from 5' to 3'.

Locus	Primer name	Sequence	Primer source
trnL intron/	*trnL*F	GGATTGAGCCTTGGTAAGGAAAC	designed in this study
trnL–F spacer	*trnF*R1	GATTTTCAGTCCTCTGCTCTACCA	designed in this study
rpl16 intron	F71	GCTATGCTTAGTGTGTGACTCGTTG	Baum *et al.* (1998)
	R1516	CCCTTCATTCTTCCTCTATGTTG	Baum *et al.* (1998)
rps16 intron	*rps*F	TGGTAGAAAGCAACGTGCGACTT	Oxelman *et al.* (1997)
	*rps*R2	TCGGGATCGAACATCAATTGCAAG	Oxelman *et al.* (1997)
ITS	17SE	ACGAATTCATGGTCCGGTGAAGTGTTCG	Sun et al. (1994)
	26SE	TAGAATTCCCCGGTTCGCTCGCCGTTAC	Sun et al. (1994)

Biosystems). Sequences for each region were aligned with MultiAlin (Corpet 1988) and ambiguously aligned regions were then manually corrected.

Phylogenetic analyses

The three cpDNA regions sequenced were combined into a single data set for phylogenetic analysis. Separate phylogenetic analyses were conducted for cpDNA and ITS data sets. In the ITS analysis, samples that showed additive nucleotide sites were not used. Insertions or deletions (Indels) in mononucleotide repeats were excluded from the alignment and the remaining informative indels were treated as a single mutational event. Site changes were weighted equally in the analyses. Phylogenetic relationships were analyzed using the maximum-parsimony (MP) method. We used MEGA version 5 (Tamura *et al.* 2011) for all analyses. The bootstrap method with 1000 replications was employed to estimate the confidence levels of monophyletic groups.

Results

Nucleotide sequences of ITS regions and phylogenetic analysis

The resultant ITS data set contained ITS1, 5.8S and ITS2 sites and comprised 786 base pairs (bp) after alignment in *Trachelospermum* (Table 3). The ITS regions of *Trachelospermum* harbored eight substitutions and no indels. Of the 23 sequenced samples, 17 resulted in a clean sequence in which single-site polymorphisms as overlapping double peaks were not detected. These 17 samples were used for phylogenetic analysis. The remaining six samples (three *Trachelospermum asiaticum* var. *asiaticum*, one *T. asiaticum* var. *majus* and two *T. jasminoides* var. *pubescens*) showed nucleotide additivity at some of the nucleotide substitution sites (Table 4). One or two additive nucleotide sites were detected in the *T. asiaticum* var. *asiaticum*-1, -6, -7 and *T. asiaticum* var. *majus*-2 samples. In *T. jasminoides* var. *pubescens*-1 and -3, additive nucleotide sites were observed at three of four nucleotide substitution sites between *T. asiaticum* and *T. jasminoides* var. *pubescens* (*T. j.* var. *pubescens*, hereafter). ITS sequences of *T. j.* var. *pubescens* without nucleotide additivity were identical to those of *T. jasminoides* var. *jasminoides* (*T. j.* var. *jasminoides*, hereafter) (Tables 4 & 5).

Intraspecific polymorphisms were detected in *Trachelospermum asiaticum* var. *asiaticum* and *T. gracilipes* var. *liukiuense* (Table 4). In *T. asiaticum* var. *asiaticum*, there were nucleotide substitutions at sequence positions 261 and 672. In *T. gracilipes* var. *liukiuense*, we detected three nucleotide substitutions between *T. gracilipes* var. *liukiuense*-1 and -2 from the Bonin Islands and *T. gracilipes* var. *liukiuense*-3 and -4 from the Ryukyu Islands.

The MP tree topologies obtained were highly concordant with morphology-based taxonomy (Fig. 2). Clade I comprised *Trachelospermum jasminoides* var. *jasminoides* and *T. j.* var. *pubescens*. Clade II included *T. asiaticum* var. *asiaticum* (*T. a.* var. *asiaticum*, hereafter), *T. asiaticum* var. *majus* (*T. a.* var. *majus*, hereafter), and *T. gracilipes* var. *liukiuense*. *Trachelospermum a.* var. *asiaticum* and var. *majus* formed a subclade (IIa) and *T. gracilipes* var. *liukiuense* formed another

TABLE 3. Comparison of phylogenetic information from variable sites for ITS and three regions of cpDNA.

Regions	Length (bp)	No. of nucleotide substitutions	No. of indels	No. of informative indels
ITS	786	8	0	0
trnL intron/ *trnL–F* spacer	870〜873	7	2	0
rpl16 intron	919〜927	7	5	2
rps16 intron	819〜826	6	2	1

TABLE 4. Variable sites of ITS sequences in *Trachelospermum*.

Sample name	ITS (Sequence position; bp)								Accession No.
	181	224	261	334	523	524	672	693	
Tracheloseprmum asiaticum var. *asiaticum*-1	A/T	A	C	T	C	C	C	A	AB710122
T. asiaticum var. *asiaticum*-2	T	A	T	T	C	C	T	A	AB710123
T. asiaticum var. *asiaticum*-3	T	A	T	T	C	C	T	A	AB610207
T. asiaticum var. *asiaticum*-4	T	A	T	T	C	C	T	A	AB710124
T. asiaticum var. *asiaticum*-5	T	A	T	T	C	C	T	A	AB710125
T. asiaticum var. *asiaticum*-6	T	A	C/T	T	C	C	C/T	A	AB710126
T. asiaticum var. *asiaticum*-7	A/T	A	C	T	C	C	C/T	A	AB710127
T. asiaticum var. *asiaticum*-8	T	A	T	T	C	C	T	A	AB710128
T. asiaticum var. *majus*-1	T	A	T	T	C	C	T	A	AB710129
T. asiaticum var. *majus*-2	T	A	C/T	T	C	C	C/T	A	AB710130
T. jasminoides var. *pubescens*-1	T	A/G	C/T	T	C	C	C/T	A	AB710131
T. jasminoides var. *pubescens*-2	T	G	C	C	C	C	C	A	AB710132
T. jasminoides var. *pubescens*-3	T	A/G	C	C/T	C	C	C/T	A	AB710133
T. jasminoides var. *pubescens*-4	T	G	C	C	C	C	C	A	AB610208
T. jasminoides var. *pubescens*-5	T	G	C	C	C	C	C	A	AB710134
T. jasminoides var. *jasminoides*-1	T	G	C	C	C	C	C	A	AB610209
T. jasminoides var. *jasminoides*-2	T	G	C	C	C	C	C	A	AB710135
T. jasminoides var. *jasminoides*-3	T	G	C	C	C	C	C	A	AB710136
T. gracilipes var. *liukiuense*-1	A	A	C	T	C	C	C	T	AB610211
T. gracilipes var. *liukiuense*-2	A	A	C	T	C	C	C	T	AB710137
T. gracilipes var. *liukiuense*-3	A	A	C	T	A	A	C	A	AB710138
T. gracilipes var. *liukiuense*-4	A	A	C	T	A	A	C	A	AB610210
Chonemorpha fragrans (outgroup)	T	A	C	C	C	C	C	A	AB710139

subclade (IIb).

Nucleotide sequences of cpDNA and phylogenetic analysis

In *Trachelospermum*, the region from the *trnL* intron to the *trnL–F* spacer (the *trnL* intron / *trnL–F* spacer) ranged from 870 to 873 bp in length, the *rpl16* intron from 919 to 927 bp, and the *rps16* intron from 819 to 826 bp (Table 3). The *trnL* intron / *trnL–F* spacer of *Trachelospermum* contained seven substitutions and two sites of indels. The *rpl16* intron included seven substitutions and five sites of indels, and the *rps16* intron contained six substitutions and two sites of indels.

In the *trnL* intron / *trnL–F* spacer and *rpl16* intron, sequences of *Trachelospermum jasminoides* var. *pubescens* were completely identical to those of *T. asiaticum*. var. *asiaticum* and *T. a.* var. *majus* (Table 5). In the *rps16* intron, intraspecific variability was detected in *T. j.* var. *pubescens*, *T. a.* var. *asiaticum*, and *T. a.* var. *majus*, respectively (Table 5, Types A & B). The same two haplotypes (A & B) were shared among *T. j.* var. *pubescens*, *T. a.* var. *asiaticum* and *T. a.* var. *majus*. Haplotype B occurred mainly in the Kinki district except for an occurrence in *T. a.* var. *asiaticum* from Yamagata Prefecture (Fig. 3). In contrast to ITS sequences, the cpDNA sequences from the *trnL* intron / *trnL–F* spacer, *rpl16* intron, and *rps16* intron showed significant differences between *T. j.* var. *jasminoides* and *T. j.* var. *pubescens* (Table 5).

The intraspecific variability of cpDNA sequences was also detected in *Trachelospermum gracilipes* var. *liukiuense* (Table 5). In all three regions of the cpDNA, we detected sequence differentiation between *T. gracilipes* var. *liukiuen-*

TABLE 5. ITS and cpDNA variations in *Trachelospermum*.

Sample name	Genotype/ Haplotype				
	ITS^{z}	*trnL* intron/ *trnL-F* spacer	*rpl16* intron	*rps16* intron	Three regions of cpDNA
Tracheloseprmum asiaticum var. *asiaticum*-1	—	A	A	B(1NS)^{y}	B(1NS)
T. asiaticum var. *asiaticum*-2	A	A	A	A	A
T. asiaticum var. *asiaticum*-3	A	A	A	A	A
T. asiaticum var. *asiaticum*-4	A	A	A	A	A
T. asiaticum var. *asiaticum*-5	A	A	A	B(1NS)	B(1NS)
T. asiaticum var. *asiaticum*-6	—	A	A	A	A
T. asiaticum var. *asiaticum*-7	—	A	A	B(1NS)	B(1NS)
T. asiaticum var. *asiaticum*-8	A	A	A	A	A
T. asiaticum var. *majus*-1	A	A	A	B(1NS)	B(1NS)
T. asiaticum var. *majus*-2	—	A	A	A	A
T. jasminoides var. *pubescens*-1	—	A	A	B(1NS)	B(1NS)
T. jasminoides var. *pubescens*-2	B(4NS)	A	A	B(1NS)	B(1NS)
T. jasminoides var. *pubescens*-3	—	A	A	B(1NS)	B(1NS)
T. jasminoides var. *pubescens*-4	B(4NS)	A	A	A	A
T. jasminoides var. *pubescens*-5	B(4NS)	A	A	A	A
T. jasminoides var. *jasminoides*-1	B(4NS)	B(6NS, 2ID)	B(4NS, 3ID)	C(2NS, 1ID)	C(12NS, 6ID)
T. jasminoides var. *jasminoides*-2	B(4NS)	B(6NS, 2ID)	B(4NS, 3ID)	C(2NS, 1ID)	C(12NS, 6ID)
T. jasminoides var. *jasminoides*-3	B(4NS)	C(5NS, 2ID)	B(4NS, 3ID)	C(2NS, 1ID)	D(11NS, 6ID)
T. gracilipes var. *liukiuense*-1	C(4NS)	D(1NS, 2ID)	C(3NS, 4ID)	D(4NS, 2ID)	E(8NS, 8ID)
T. gracilipes var. *liukiuense*-2	C(4NS)	D(1NS, 2ID)	C(3NS, 4ID)	D(4NS, 2ID)	E(8NS, 8ID)
T. gracilipes var. *liukiuense*-3	D(5NS)	E(2NS, 2ID)	D(4NS, 3ID)	E(1NS)	F(7NS, 5ID)
T. gracilipes var. *liukiuense*-4	D(5NS)	E(2NS, 2ID)	E(4NS, 3ID)	E(1NS)	F(7NS, 5ID)
Chonemorpha fragrans (outgroup)	Out(44NS, 2ID)	Out(11NS, 5ID)	Out(8NS, 5ID)	Out(9NS, 3ID)	Out (28NS, 13ID)

z A dash (—) indicates that the haplotype has not been determined because the sequence showed nucleotide additivity.

y The number of variable sites relative to the A-type sequence or haplotype A is shown; NS, Nucleotide substitution; ID, Insertion/ Deletion.

se-1 and -2 from the Bonin Islands and *T. gracilipes* var. *liukiuense*-3 and -4 from the Ryukyu Islands.

The monophyly of each species in the ITS tree was not supported in the cpDNA tree (Fig. 4). Clade I was composed of three subclades (Ia, Ib, & Ic). *Trachelospermum asiaticum* var. *asiaticum*, *T. a* var. *majus* and *T. jasminoides* var. *pubescens* were lumped together in subclade Ia. *Trachelospermum j.* var. *jasminoides* and *T. gracilipes* var. *liukiuense* from the Ryukyu Islands formed the remaining two subclades (Ib & Ic), respectively. Clade II only included *T. gracilipes* var. *liukiuense* from the Bonin Islands.

Discussion

Natural hybridization between Trachelospermum jasminoides *var.* pubescens *and* T. asiaticum

In ITS sequences of *Trachelospermum jasminoides* var. *pubescens*-1 from Kyoto and *T. j.* var. *pubescens*-3 from Wakayama, additive nucleotide sites, adenine/ guanine or thymine/ cytosine, were shown at three of the four nucleotide substitution sites detected between *T. asiaticum* and *T. jasminoides* (Table 4). The ITS sequences with the additive state of the parental sequences for

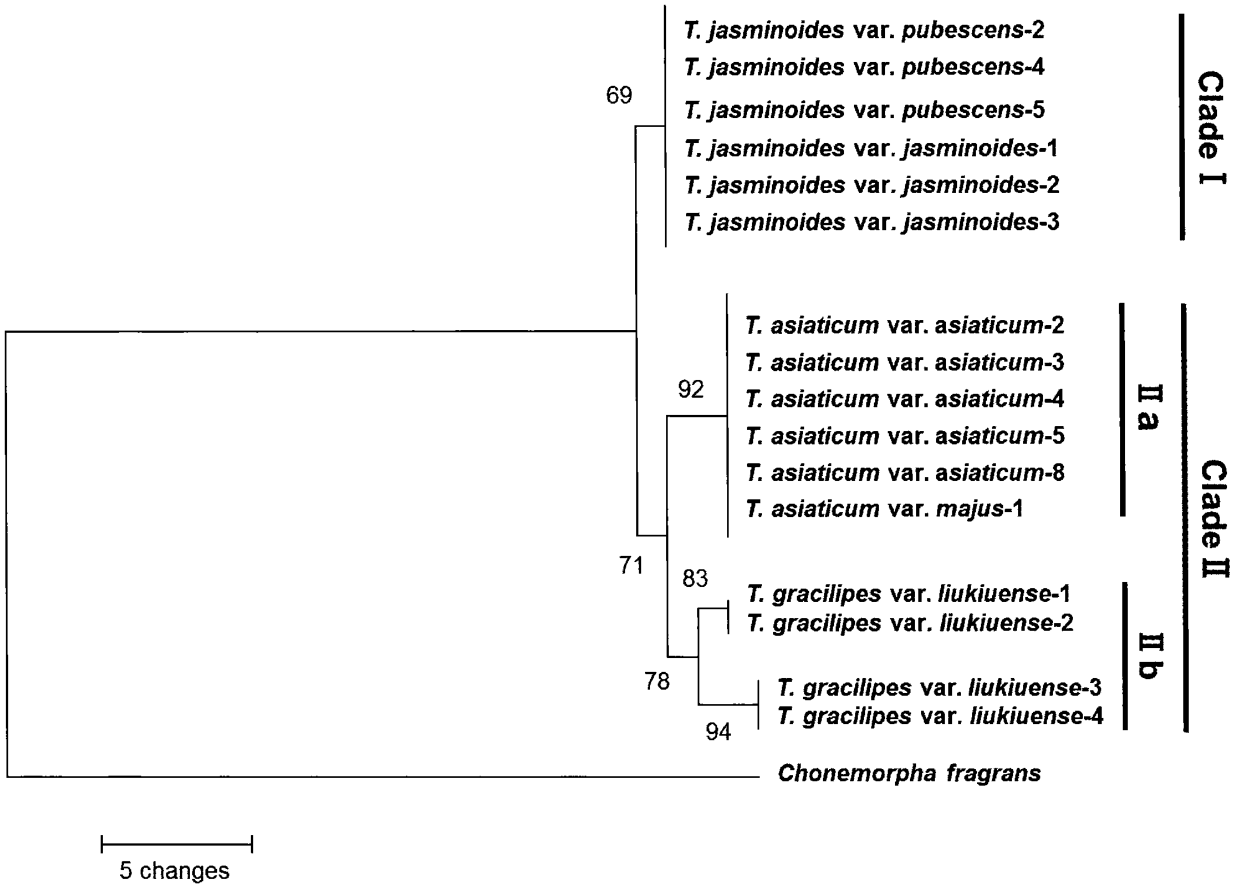
T. jasminoides var. pubescens-2
T. jasminoides var. pubescens-4
T. jasminoides var. pubescens-5
T. jasminoides var. jasminoides-1
T. jasminoides var. jasminoides-2
T. jasminoides var. jasminoides-3
T. asiaticum var. asiaticum-2
T. asiaticum var. asiaticum-3
T. asiaticum var. asiaticum-4
T. asiaticum var. asiaticum-5
T. asiaticum var. asiaticum-8
T. asiaticum var. majus-1
T. gracilipes var. liukiuense-1
T. gracilipes var. liukiuense-2
T. gracilipes var. liukiuense-3
T. gracilipes var. liukiuense-4
Chonemorpha fragrans
69
92
71
83
78
94
Clade I
II a
II b
Clade II
5 changes

FIG. 2. Strict consensus tree resulting from maximum parsimony analysis of ITS sequences. Numbers on branches indicate bootstrap values in percentages based on 1,000 replicates. Branch lengths correspond to the number of nucleotide substitutions.

hybrids have been reported in *Paeonia* (Sang *et al.* 1995), *Armeria* (Aguilar *et al.* 1999), *Glycine* (Rauscher *et al.* 2004), ×*Crepidiastrixeris* (Saito *et al.* 2006), and *Schoenoplectus* (Yano *et al.* 2010). These phenomena suggest that ITS sequences in hybrids may reflect their origin from different parental species (Yano *et al.* 2010). In our study, the ITS sequence data suggested that *T. j.* var. *pubescens*-1 and *T. j.* var. *pubescens*-3 were hybrids between *T. asiaticum* and *T. j.* var. *pubescens*. However, sequence positions 334 in *T. j.* var. *pubescens*-1 and 261 in *T. j.* var. *pubescens*-3 did not show additivity, in spite of the fact that they were nucleotide substitution sites between *T. asiaticum* and *T. j.* var. *pubescens*. The results suggest that *T. asiaticum* has nucleotide polymorphisms at each site, as Yano *et al.* (2010) described in *Schoenoplectus hotarui.*

Trachelospermum asiaticum is widely distributed westward from Iwate Prefecture. Therefore, *T. jasminoides* var. *pubescens*, which is distributed westward from Kinki district occurs sympatrically with *T. asiaticum*. Both *T. asiaticum* and *T. j.* var. *pubescens* have been found in Kyoto city (Uemachi & Shimomura 2008). The results support the probability that *T. j.* var. *pubescens*-1 from Kyoto and *T. j.* var. *pubescens*-3 from Wakayama are natural hybrids between *T. asiaticum* and *T. j.* var. *pubescens*.

ITS polymorphisms resulting from hybridization are considered to be homogenized by backcrossing or concerted evolution (Sang *et al.* 1995, Aguilar *et al.* 1999, Rauscher *et al.* 2004, Yamaji *et al.* 2007a). According to Aguilar *et al.* (1999),

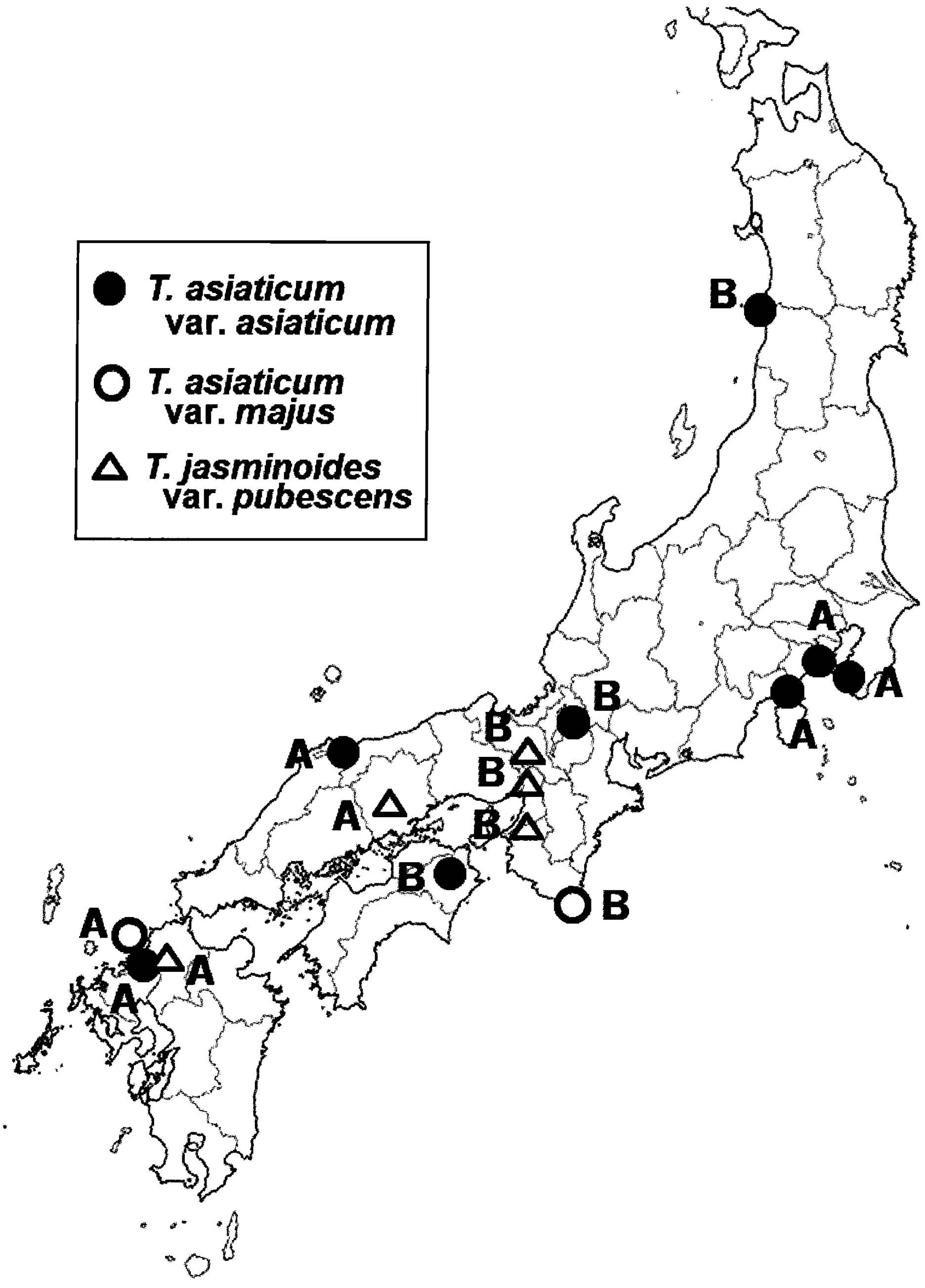
● T. asiaticum var. asiaticum
○ T. asiaticum var. majus
△ T. jasminoides var. pubescens
A
B

FIG. 3. Geographical distribution of the cpDNA haplotypes in *Trachelospermum asiaticum* and *T. jasminoides* var. *pubescens.*

the homogenization of polymorphisms by the effects of concerted evolution has been observed in the F_2 of artificial hybrids between two species of *Armeria.* Moreover, direct sequencing analysis has limits in recognizing additive nucleotide sites (Yamaji *et al.* 2007b). Cloning of PCR products and sequencing of multiple clones are effective at revealing ITS nucleotide polymorphisms (Yamaji *et al.* 2007b). It is therefore possible that our samples with non-additive states harbor intragenomic polymorphisms. Further cloning analyses are needed to construct more reliable ITS phylogenetic trees and to more precisely detect hybridization events between *Trachelospermum asiaticum* and *T. jasminoides* var. *pubescens.*

Distribution pattern of cpDNA haplotypes

In the combined cpDNA sequences, haplo-

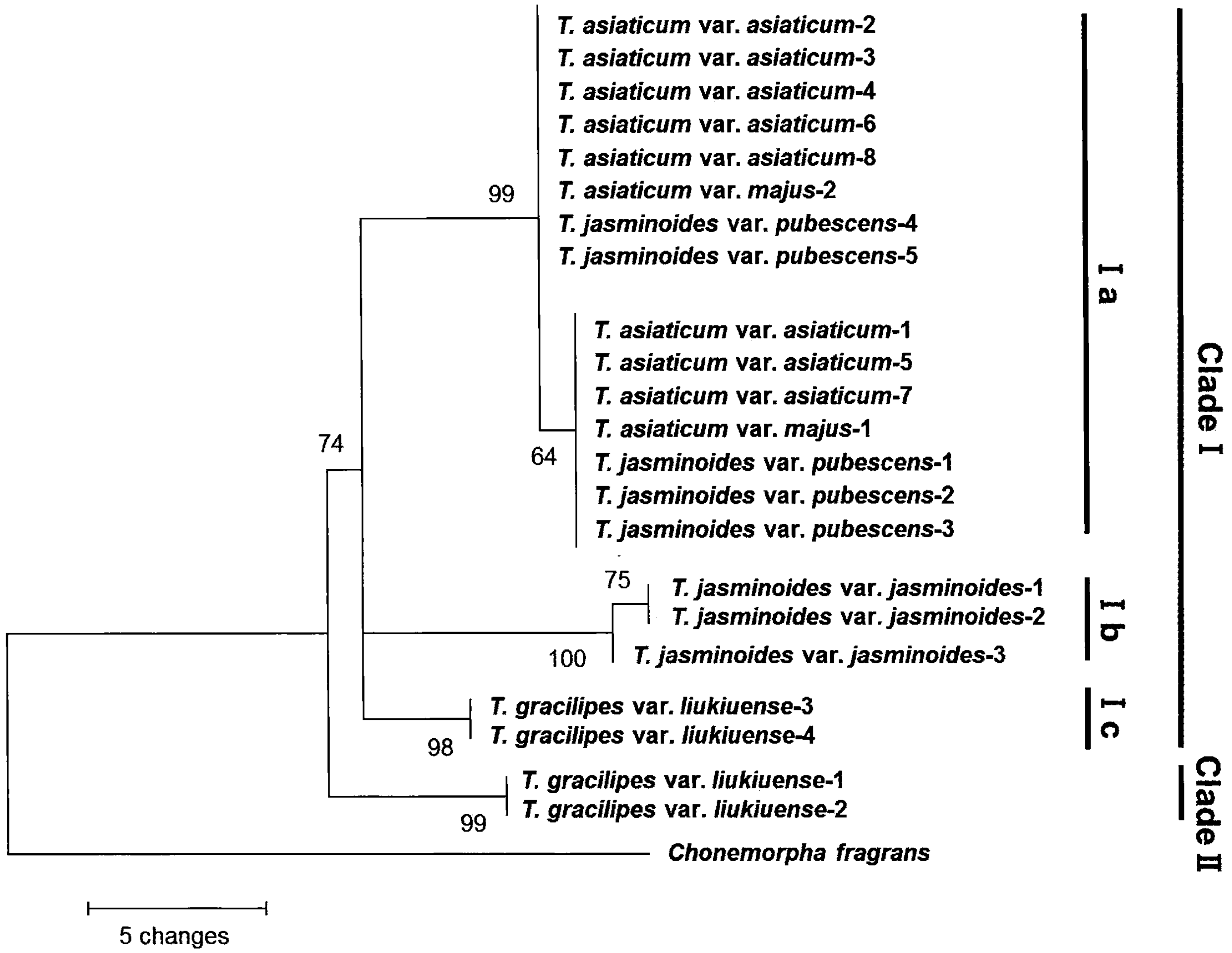
T. asiaticum var. *asiaticum*-2
T. asiaticum var. *asiaticum*-3
T. asiaticum var. *asiaticum*-4
T. asiaticum var. *asiaticum*-6
T. asiaticum var. *asiaticum*-8
T. asiaticum var. *majus*-2
T. jasminoides var. *pubescens*-4
T. jasminoides var. *pubescens*-5
T. asiaticum var. *asiaticum*-1
T. asiaticum var. *asiaticum*-5
T. asiaticum var. *asiaticum*-7
T. asiaticum var. *majus*-1
T. jasminoides var. *pubescens*-1
T. jasminoides var. *pubescens*-2
T. jasminoides var. *pubescens*-3
T. jasminoides var. *jasminoides*-1
T. jasminoides var. *jasminoides*-2
T. jasminoides var. *jasminoides*-3
T. gracilipes var. *liukiuense*-3
T. gracilipes var. *liukiuense*-4
T. gracilipes var. *liukiuense*-1
T. gracilipes var. *liukiuense*-2
Chonemorpha fragrans
99
64
74
75
100
98
99
I a
I b
I c
Clade I
Clade II
5 changes

FIG. 4. Strict consensus tree resulting from maximum parsimony analysis of cpDNA sequences. Numbers on branches indicate bootstrap values in percentages based on 1,000 replicates. Branch lengths correspond to the number of nucleotide substitutions and informative indels.

types A and B were shared among *Trachelospermum jasminoides* var. *pubescens*, *T. asiaticum* var. *asiaticum* and *T. a.* var. *majus* (Table 5). Interestingly, the two haplotypes (A & B) were geographically localized, with proximate samples of different taxa tending to have the same haplotype (Fig. 3). The presence of geographical structures in cpDNA haplotypes across different taxa has been reported for many plant species and is often interpreted as a consequence of hybridization and introgression (e.g., Whittemore & Schaal 1991, Ohi *et al.* 2003, Kanno *et al.* 2004, Watanabe *et al.* 2006,). In the present study, the geographical structure of cpDNA in Japanese *Trachelospermum* was suggested, although a more detailed study using more samples will be needed to clarify the geographic boundary between haplotypes A and B.

Inconsistencies between the ITS and cpDNA phylogenetic trees

Our results showed that the ITS sequences of *Trachelospermum jasminoides* var. *pubescens* were identical to those of *T. j.* var. *jasminoides* and were clearly different from those of *T. asiaticum* (Table 5). In contrast, the sequences of the cpDNA regions in *T. j.* var. *pubescens* and *T. asiaticum* were the same, but clearly different from those in *T. j.* var. *jasminoides*. We found that the cpDNA phylogeny did not agree with morphology-based taxonomy, whereas the ITS phylogenies agreed with morphology to some degree (Figs. 2

& 4).

Inconsistencies between the phylogenetic trees using cytoplasmic and nuclear genes have been explained by incomplete lineage sorting (Mason-Gamer *et al.*1995, Linder & Rieseberg 2004, Jakob & Blattner 2006) or chloroplast capture (Rieseberg *et al.* 1991, Rieseberg & Soltis 1991, Wolfe & Elisens 1995, Rieseberg *et al.* 1996, Tsitrone *et al.* 2003). Incomplete lineage sorting is caused by the persistence of ancestral polymorphisms through speciation events, whereas chloroplast capture is caused by the introgression of chloroplasts from one species into another. Many cases of chloroplast capture have been reported in various taxa such as *Helianthus*, *Quercus*, *Penstemon*, *Mitella* and *Asiasarum* (Rieseberg *et al.* 1991, Wolfe & Elisens 1995, Okuyama *et al.* 2005, Yamaji *et al.* 2007a). It is difficult, however, to distinguish introgression from incomplete lineage sorting in phylogenic analyses (Jakob & Blattner 2006, Joly *et al.* 2009).

In this study, *Trachelospermum jasminoides* var. *pubescens* contained the same ITS genotype as *T. j.* var. *jasminoides* and the same cpDNA haplotypes as *T. asiaticum* (Table 5). Moreover, the cpDNA haplotypes (A and B) shared by *T. j.* var. *pubescens* and *T. asiaticum* were geographically localized regardless of the taxa (Fig. 3). As mentioned above, haplotype sharing across sympatric taxa has been explained by gene flow among sympatric taxa (Whittemore & Schaal 1991, Yamaji *et al.* 2007a). Moreover, our ITS sequence data suggested natural hybridization between *T. j.* var. *pubescens* and *T. asiaticum* (Table 4). Although we cannot completely exclude incomplete lineage sorting as contributing to incongruence between the phylogenetic trees of ITS and cpDNA, our findings show that chloroplast capture was more likely to be the cause of the results observed. Given that chloroplast capture has occurred in *Trachelospermum*, the nonmonophyly of *T. jasminoides* in the cpDNA tree can be explained by hypothesizing that *T. j.* var. *pubescens* may have obtained its cpDNA from *T. asiaticum*.

Intraspecific differences in Trachelospermum asiaticum

Trachelospermum asiaticum var. *majus* has larger leaves than *T. a.* var. *asiaticum* and grows in coastal areas on the Pacific side of Honshu westward from Chiba Prefecture and along the coasts of Shikoku and Kyushu (Kitamura & Murata 1971, Satake *et al.* 1989). In our study, sequence differentiation between *T. a.* var. *majus* and *T. a.* var. *asiaticum* was not detected in either the ITS or cpDNA analysis. It appears that *T. a.* var. *majus* has adapted to the environmental conditions of coastal areas without conspicuous sequence differentiation. *Trachelospermum a.* var. *majus* can therefore be recognized as a coastal ecotype.

Sequence differentiation between Trachelospermum gracilipes *var.* liukiuense *in the Bonin Islands and the Ryukyu Islands*

Our molecular data indicated sequence differentiation between *Trachelospermum gracilipes* var. *liukiuense* from the Bonin Islands and Ryukyu Islands in both the ITS and cpDNA analyses (Table 5). The monophyly of the two groups was not supported in the MP tree of cpDNA (Fig. 4). In our study, we used only two individuals from Hahajima in the Bonin Islands and only two individuals from Okinawajima in the Ryukyu Islands. Further molecular analyses using *T. gracilipes* var. *liukiuense* from other islands in the Ryukyu Islands and Bonin Islands are therefore needed to confirm the genetic differentiation in *T. gracilipes* var. *liukiuense*. In China and Taiwan, *T. gracilipes* and *T. asiaticum* are not distinguished from each other (Jussieu 1995, Li & Huang 1998). Further studies using *T. gracilipes*, *T. asiaticum*, and related species from Taiwan, China, and Southeast Asia are needed to clarify the relationships among these closely related taxa.

Hatsusima (1940) reported *Trachelospermum foetidum* (*T. gracilipes* var. *liukiuense* from the Bonin Islands) as having much larger sepals, much larger flowers, and broader flower buds than *T. liukiuense* (*T. gracilipes* var. *liukiuense* from the Ryukyu Islands). We need to re-examine the morphological characteristics of *T. gracilipes* var. *liukiuense* from the Bonin Islands and Ryukyu Islands to be able to discuss the genetic differentiation between the two groups.

Although further precise studies, including morphological analyses, are needed, the present study indicates sequence differentiation in ITS and cpDNA between *Trachelospermum gracilipes* var. *liukiuense* from the Bonin Islands and from the Ryukyu Islands. From the viewpoint of conservation biology, the results suggest that *T. gracilipes* var. *liukiuense* of the Bonin Islands should be distinguished from the plants of the Ryukyu Islands, and deserves special conservation consideration equal to that given to other species endemic to the Bonin Islands.

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